0022-202X/81/7602-0133$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 76:133–136, 1981

Vol. 76, No. 2
Printed in U.S.A.

# *In Vitro* DNA Synthesis in Lymphocytes from Guinea Pigs Epicutaneously Sensitized with DNCB

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**Lymph node lymphocytes from guinea pigs sensitized with the hapten homogenized in Freund's complete adjuvant or pretreated with cyclophosphamide shortly before an epicutaneous sensitization are capable of responding *in vitro* to a larger set of antigenic preparations than the lymphocytes from only epicutaneously sensitized animals.**

**This may be due to a formation of a larger set of antigenic determinants when the hapten was homogenized in Freund's complete adjuvant or to a formation of a larger repertoire of lymphocytes capable of recognizing these determinants as it might be the result of pretreatment with cyclophosphamide. These procedures may also change the proportion of responding cells in the lymphocyte suspensions.**

The experimental model of guinea pig contact sensitivity has been widely used both for research of the mechanism of allergic eczema and for investigation of potential allergenicity of various substances. For this purpose animals were sensitized either by an epicutaneous application of the hapten or by intradermal injections of the hapten homogenized in Freund's complete adjuvant [1]. However, some experimental results have shown that there exist substantial differences in contact sensitivity induced by these 2 sensitising methods. These differences become manifest both *in vivo* and *in vitro*.

The main *in vivo* difference consists in the intensity of the eliciting skin reaction and in the composition of the cellular infiltrate. It has been shown that contact sensitivity skin reactions in guinea pigs sensitized by the epicutaneous route are less erythematous and less indurated but contain more basophils than the skin reaction in animals sensitized with the antigen in Freund's complete adjuvant [2].

In a previous paper it has been demonstrated that several antigenic preparations such as dinitrophenyl (DNP)-coated nonviable lymphocytes (DNCB-Ly) or DNP-modified macrophages (DNP-M$\phi$) are capable of stimulating *in vitro* lymphocytes from guinea pigs sensitized with dinitrofluorobenzene (DNFB) in Freund's complete adjuvant (FCA). Enhanced DNA-synthesis in vitro was also achieved by a short pulse of sensitized lymphocytes with dinitrobenzenesulfonic acid sodium salt ($DNBSO_3Na$) and to a lesser extent also by a direct addition of this hapten or hapten-autologous protein conjugate to the culture [3].

Manuscript received May 28, 1980; accepted for publication August 8, 1980.

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Abbreviations:
CY: Cytimun, a cyclophosphamide preparation
DMSO: dimethylsulfoxid
DNCB: 2,4 dinitrochlorobenzene
DNCB-Ly: dinitrophenyl (DNP)-coated non viable lymphocytes
DNFB: 2,4 dinitrofluorobenzene
$DNBSO_3Na$: 2,4 dinitrobenzene sulfonic acid sodium salt
DNP-M$\phi$: DNP-modified macrophages
FCA: Freund's complete adjuvant
$SO_3$-Ly: $DNBSO_3Na$ pulsed viable lymphocytes

In vitro stimulation of lymphocytes from guinea pigs sensitized by an epicutaneous application of the hapten dinitrochlorobenzene (DNCB) was, however, achieved only when DNP-modified macrophages were used as antigen [4]. All the other antigenic preparations which were successful in stimulating lymphocytes from animals sensitized with the help of FCA failed to induce an enhanced DNA-synthesis in lymphocytes from animals sensitized by the epicutaneous method [5].

In the present paper it is shown that lymphocytes from guinea pigs sensitized either intradermally with the hapten homogenized in Freund's complete adjuvant or pretreated with cyclophosphamide shortly before sensitization are capable of responding *in vitro* to a larger set of specific antigenic preparations than lymphocytes from animals sensitized epicutaneously. On the basis of the literature and of our experiments attempt is made to clarify the mechanism of these results.

## MATERIALS AND METHODS

### *Animals*

Inbred strain 13 guinea pigs of either sex bred at the Institute for Biochemical Research, Füllinsdorf, Switzerland, weighing 350–450 gm, were used. They were fed on pellet diet supplemented *ad libitum* with water containing vitamin C.

Immunization. Guinea pigs were sensitized either by epicutaneous application of 25 $\mu$l of a 50% solution of DNCB (2,4-dinitrochlorobenzene, Merck, Darmstadt, FRG) in acetone on the nape of the neck and on the ears or by 5 intradermal injections of totally 500 $\mu$g DNFB (2,4-dinitrofluorobenzene, Fluka AG, Buchs SG, Switzerland) homogenized in 0.5 ml FCA (Freund's complete adjuvant, Difco Lab., Detroit, Mich., USA) into the footpads and the nape of the neck.

CY (Cyclophosphamide derivative Cytimun C 682, Asta-Werke AG, Brackwede, FRG) was given intraperitoneally (250 mg/kg) 3 days before the sensitizing procedure.

The split adjuvant technique consisted of a simultaneous epicutaneous application of DNCB and intradermal injections of FCA. A separate group of animals was injected i.d. with FCA only.

Harvesting of lymphoid cells. A single cell suspension was prepared from draining lymph nodes. The cells were teased from the nodes with specially constructed forks and filtered through nylon stocking mesh.

T- and B-cells were separated on 50 ml syringes filled with nylon wool (Leuko-Pak, Fenwal Lab., Deerfield, Ill., USA). The nonadherent T-cells were collected by eluting the column with RPMI 1640 medium containing 10% fetal calf serum (FCS) after incubation of the lymphocytes for 30 min at 37°C. B-cells were recovered by repeatedly compressing the nylon wool with forceps and rinsing the column with RPMI 1640 medium containing 5% FCS. Contaminating adherent T-cells were removed by rosetting them with papain-treated rabbit erythrocytes and centrifuging them through a Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) cushion (density 1.085 g/ml, 15 min, 1500 $\times g$).

Antigenic preparations. $SO_3$-Ly were prepared by pulsing sensitized lymphocytes for 2 hr with 500 $\mu$g/ml $DNBSO_3Na$ (2,4-dinitrobenzenesulphonic acid sodium salt, Eastman Kodak, Rochester, N.Y., USA). DNCB-Ly ($7 \times 10^4$/ml) were obtained by incubating blood lymphocytes, purified on a Ficoll-Paque cushion, in a 10% DNCB solution in DMSO (dimethylsulfoxide, Schuchardt, Hohenbrunn, FRG) for 30 min at 37°C. DNP-M$\phi$ ($5 \times 10^5$/ml) were prepared by incubating irradiated (3300 r) peritoneal exudate cells, harvested 3–5 days after an i.p. injection of 30 ml light paraffin oil (Drakeol 6-VR White, Penna, Butler, Penn. USA), in a DNFB solution (10 $\mu$g/ml) in Hanks' BSS (GIBCO Bio-Cult, Glasgow, Scotland) for 30 min at 37°C.

*In vitro* stimulation. $5 \times 10^5$ lymphocytes were cultured in 1 ml RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 5

μg/ml 5-fluorocytosine (Hoffmann-La Roche & Co., Basle, Switzerland), 50 μg/ml gentamicin (Schering & Co., Kenilworth, N.J.) and $10^{-5}$ M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) for 120 hr at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. The culture was pulsed for the last 18 hr with 1 μCi of $^3$H-thymidine (TRA 120, spec. act. 5 Ci/mmol, The Radiochemical Centre, Amersham, England) per tube (12 × 75 mm, round-bottom plastic tubes, Flacon, Oxnard, Calif.) and DNA synthesis was determined by precipitating TCA-insoluble material on glass fiber filters (Whatman Inc., Clifton, N.J.) and by counting in a liquid scintillation spectrometer.

## RESULTS

In table I it is shown that lymphocytes from guinea pigs sensitized with DNFB in FCA responded to a short pulse with the cross-reacting hapten $DNBSO_3$ Na (group 2) as well as to a continuous stimulation with either DNP-coated nonviable syngeneic blood lymphocytes (DNCB-Ly) (group 4) or with DNP-modified macrophages (DNP-Mϕ) (group 6) by an increased *in vitro* DNA-synthesis. DNP-modified macrophages were the most efficient stimulators. On the other hand, lymphocytes from animals sensitized epicutaneously without the help of FCA responded only to DNP-modified macrophages (group 5) whereas a short pulse with $DNBSO_3$ Na or use of DNP-coated nonviable lymphocytes did not elicit any *in vitro* response.

Addition of 50% lymph node lymphocytes from DNCB epicutaneously sensitized animals to lymphocytes from guinea pigs sensitized with the hapten homogenized in FCA and stimulated *in vitro* with DNCB-Ly (group 7) did not suppress the *in vitro* response of the latter.

From the results presented in Table II it is evident, that only lymphocytes from guinea pigs sensitized intradermally with DNFB homogenized in FCA were capable of responding *in vitro* to DNP-coated lymphocytes (group 2). Lymphocytes from guinea pigs sensitized by a simultaneous application of FCA intradermally and DNCB epicutaneously (split adjuvant technique) were unable to respond *in vitro* to this antigenic preparation (group 3). Moreover, a co-culture of lymphocytes from animals sensitized with FCA, i.d., with lymphocytes from guinea pigs sensitized epicutaneously with DNCB did not render the latter responsive to the mentioned antigenic stimulation (group 4).

In order to find out whether the unresponsiveness of lymphocytes from DNCB, e.c., sensitized animals to a short pulse with the hapten ($DNBSO_3$ Na) is due to lack of normal macrophages (macrophage help) these cells were added to the culture after the pulse with the hapten. As a control the same number of hapten modified macrophages ($5 \times 10^5$) or a combination of $10^3$ DNP-Mϕ with $4 \times 10^4$ normal macrophages were used as stimulators. The results in Table III demonstrate that the modification of the macrophages by the hapten has to occur prior to their addition to the lymphocyte culture (group 3). A simple addition of normal macrophages to a culture of $DNBSO_3$ Na pulsed lymphocytes from DNCB e.c. sensitized guinea pigs did not elicit any *in vitro* response (group 2).

Finally, it is shown, that elimination of B-cells from the lymphocyte suspension from DNCB e.c. sensitized animals (Table IV) did not render the remaining T-cells responsive to the stimulation with an antigenic preparation otherwise ineffective in unseparated lymphocyte suspensions (DNP-coated lymphocytes). However, pretreatment of guinea pigs with cyclophosphamide shortly before sensitization induced in these animals a population of lymphocytes capable of responding *in vitro* to the mentioned antigenic preparation. Both unseparated and T-lymphocytes were equally responsive whereas B-cells remained unresponsive in spite of the CY-treatment.

TABLE I. *In vitro DNA-synthesis of lymphocytes from guinea pigs sensitized either epicutaneously with DNCB or intradermally with DNFB homogenized in FCA*

| Group | Sensitization | No. of animals | *In vitro* challenge | Baseline cpm | Antigen cpm | S.I. |
|---|---|---|---|---|---|---|
| 1 | DNCB e.c. | 8 | $DNBSO_3Na$ 500 μg/ml 2 h pulse | 3882 ± 1207 | 4358 ± 954 | 1.4 ± 0.4 |
| 2 | DNFB/FCA i.d. | 7 | –"– | 1821 ± 350 | 24998 ± 5100 | 13.7 ± 6.9 |
| 3 | DNCB e.c. | 16 | DNCB-Ly $7 \times 10^4$ | 3440 ± 311 | 5100 ± 593 | 1.6 ± 0.3 |
| 4 | DNFB/FCA i.d. | 18 | –"– | 1460 ± 196 | 17082 ± 1995 | 11.5 ± 7.3 |
| 5 | DNCB e.c. | 24 | DNP-Mϕ $5 \times 10^5$ | 1846 ± 279 | 28933 ± 5762 | 24.6 ± 6.8 |
| 6 | DNFB/FCA i.d. | 12 | –"– | 2641 ± 612 | 71307 ± 8341 | 27.4 ± 13.4 |
| 7 | DNFB/FCA i.d.-Ly + 50% DNCB e.c.-Ly | 6 | DNCB-Ly $7 \times 10^4$ | 986 ± 82 | 10352 ± 950 | 10.5 ± 3.5 |

Lymph node lymphocytes from guinea pigs sensitized either e.c. with DNCB (groups 1, 3 and 5) or i.d. with DNFB in FCA (groups 2, 4 and 6) were pulsed for 2 hr with $DNBSO_3Na$ (groups 1 and 2) before the 120 hr culture or stimulated during the whole culture time with DNP-coupled nonviable lymphocytes (groups 3 and 4). In groups 5 and 6 DNP-modified macrophages were used as stimulators. In group 7 the same set up as in group 4 was used but $2.5 \times 10^5$ lymph node lymphocytes from DNCB e.c. sensitized guinea pigs were added at the beginning of the culture. Non-$DNBSO_3Na$-treated viable lymphocytes or nonviable unconjugated lymphocytes or normal macrophages served as controls. Results are expressed as mean cpm ± standard error and as stimulation indices (S.I.).

TABLE II. *Effect of Freund's complete adjuvant (FCA) on* in vitro *DNA synthesis of lymphocytes from DNCB-sensitized guinea pigs*

| Group | Sensitization | No. of animals | Co-Ly cpm | DNCB-Ly cpm | S.I. |
|---|---|---|---|---|---|
| 1 | DNCB e.c. | 16 | 3440 ± 311 | 5100 ± 593 | 1.6 ± 0.3 |
| 2 | DNFB/FCA i.d. homogenized | 10 | 1460 ± 196 | 39420 ± 3991 | 27.4 ± 4.3 |
| 3 | DNCB e.c. FCA i.d. | 10 | 766 ± 106 | 1759 ± 414 | 2.3 ± 0.4 |
| 4 | LNLy DNCB e.c. LNLy FCA i.d. | 8 | 2979 ± 930 | 4056 ± 1122 | 1.7 ± 0.2 |

Lymph node lymphocytes ($5 \times 10^5$/ml RPMI 1640) from guinea pigs sensitized either with DNCB e.c. (group 1) or with DNFB homogenized in FCA (group 2) were cultured with $7 \times 10^4$ DNCB-conjugated nonviable lymphocytes (DNCB-Ly) or with the same number of control (DMSO-treated) lymphocytes (Co-Ly). In group 3 animals were simultaneously sensitized with DNCB e.c. and FCA i.d. In the last group (group 4) $2.5 \times 10^5$ lymphocytes from DNCB e.c. sensitized animals were mixed with equal numbers of lymphocytes from FCA i.d. sensitized guinea pigs and cultured as above. Results are expressed as mean cpm ± standard error and as stimulation indices (SI).

TABLE III. *Effect of macrophages on* **in vitro** *DNA synthesis of lymphocytes from DNCB e.c. sensitized guinea pigs*

| Group | *In vitro* challenge | No. of animals | Baseline cpm | Antigen cpm | S.I. |
|---|---|---|---|---|---|
| 1 | $DNBSO_3Na$ | 8 | 3882 ± 1207 | 4385 ± 954 | 1.4 ± 0.4 |
| 2 | $DNBSO_3Na$ + $5 \times 10^5$ NMϕ | 8 | 2860 ± 361 | 3231 ± 551 | 1.3 ± 0.3 |
| 3 | DNP-Mϕ $5 \times 10^5$ | 16 | 1846 ± 279 | 28933 ± 5762 | 24.6 ± 6.8 |
| 4 | DNP-Mϕ $10^3$ + NMϕ $4 \times 10^4$ | 4 | 1221 ± 180 | 7884 ± 2824 | 6.5 ± 2.4 |

Lymph node lymphocytes from DNCB e.c. sensitized guinea pigs were pulsed for 2 hr with 500 μg/ml $DNBSO_3Na$ and $5 \times 10^5$ cells treated ($SO_3Ly$) or nontreated (NLy) were cultured with (group 2) or without (group 1) addition of $5 \times 10^5$ normal macrophages (NMϕ). In group 3 lymphocytes were not pulsed with $DNBSO_3Na$, but $5 \times 10^5$ normal (NMϕ) or DNP-modified (DNP-mϕ) macrophages were added to the culture. In the group 4 DNP-Mϕ ($10^3$) + NMϕ ($4 \times 10^4$) were used. Results were expressed as in Table II.

TABLE IV. In vitro *DNA synthesis of lymphocytes from DNCB e.c. sensitized guinea pigs. Effect of pretreatment with cyclophosphamide (CY)*

| Group | Lymphocytes | Treatment | No. of animals | Co-Ly cpm | DNCB-Ly cpm | S.I. |
|---|---|---|---|---|---|---|
| 1 | Unseparated | — | 20 | 2341 ± 207 | 3558 ± 409 | 1.5 ± 0.2 |
| 2 | | CY | 19 | 3211 ± 623 | 51697 ± 7488 | 16.1 ± 3.6 |
| 3 | T-cells | — | 9 | 1428 ± 187 | 3141 ± 521 | 2.2 ± 0.4 |
| 4 | | CY | 14 | 841 ± 225 | 10176 ± 3926 | 12.1 ± 5.1 |
| 5 | B-cells | — | 5 | 4431 ± 1981 | 5317 ± 2039 | 1.2 ± 0.2 |
| 6 | | CY | 5 | 4341 ± 1431 | 3473 ± 724 | 0.8 ± 0.1 |

Lymph node lymphocytes from guinea pigs sensitized epicutaneously with dinitrochlorobenzene (DNCB) and either pretreated 3 days before sensitization with cyclophosphamide (CY) or sensitized only (—) were separated into T- and B-cells as described in "Material and Methods." To $5 \times 10^5$ cells in 1 ml RPMI 1640 were added $7 \times 10^4$ DNCB-conjugated nonviable lymphocytes (DNCB-Ly) or an equal number of control (DMSO-treated) cells (Co-Ly). Results are expressed as mean cpm ± standard error and as stimulation indices (SI).

Lymph node lymphocytes were usually harvested 10–14 days after sensitizing procedure but the stimulation indices of unseparated lymphocytes from CY-treated guinea pigs were about the same magnitude (14.3 ± 4.2) when cells were harvested 7 to 10 days later (20 to 24 days after CY-treatment).

## DISCUSSION

The results in this study indicate that there exists a substantial difference between contact sensitivity induced in guinea pigs by the application of the hapten epicutaneously without or intradermally with the help of adjuvant. Two antigenic preparations, namely a short pulse of sensitized lymphocytes with the hapten and a stimulation with DNP-coated nonviable blood lymphocytes, are unable to induce a positive response in lymphocytes from DNCB-epicutaneously sensitized guinea pigs in the *in vitro* DNA-synthesis assay. DNFB-conjugated guinea pig skin extracts also failed in this respect [5].

In other species such as in man or in the mouse a positive response was, however, achieved (6, 8, 9, 10, 11, 12).

Lymphocytes from guinea pigs exhibited an enhanced *in vitro* synthesis when (a) animals were sensitized intradermally with the hapten homogenized in FCA; (b) epicutaneously sensitized animals were pretreated with cyclophosphamide shortly before sensitization; (c) hapten modified macrophages were used.

It is known that contact sensitivity is a hypersensitivity directed against a set of antigens induced in *in vivo* conjugation of one and the same hapten to various autologous proteins [13]. If *in vitro* prepared conjugates are used for elicitation of *in vivo* reactions, these reactions are always of lower intensity or do not occur at all.

Generally the effect of adjuvants on the immune response is a very complex one and affects both the antigen-reactive and the auxiliary cells as well as the function of the antigens [14]. The numbers of macrophages and T-cells increases, their circulation becomes enhanced and their surface markers (Ia-antigen, Fc-receptor) are changed. Furthermore, the dissemination of the antigen and its uptake by macrophages is also changed.

The following considerations may help to explain the mechanism of the effect of FCA in our experiments.

Lymphocytes obtained from guinea pigs sensitized by the split adjuvant method responded to a smaller set of antigenic preparations than lymphocytes from animals sensitized with the hapten homogenized in FCA.

Co-cultures of lymphocytes from FCA-sensitized guinea pigs with lymphocytes from animals sensitized epicutaneously with the hapten did not enlarge the set of antigenic preparations to which the latter were able to respond *in vitro*.

Both these results made improbable a nonspecific activation of antigen reactive and/or auxiliary cells by FCA as the mechanism of the FCA effect in our system. It appears more probable that in our system FCA reacts directly with the hapten thus forming a larger set of antigenic determinants. Under these conditions a larger repertoire of DNP-specific lymphocytes is activated which consequently are capable of reacting *in vitro* with a larger set of antigenic preparations.

The haptenization of macrophages leads to formation of new epitopes which are capable of stimulating lymphocytes from DNCB epicutaneously sensitized animals. This may be due either to a larger set of determinants formed on macrophages or to their closer similarity to determinants formed *in vivo* by the epicutaneous application of the hapten. An addition of nonhaptenized macrophages to lymphocytes from epicutaneously sensitized animals pulsed with the hapten, did not effectuate enhanced DNA-synthesis. It is suggested that by the short pulse with the hapten mainly T-cells are haptenized. For a significant *in vitro* stimulation of sensitized lymphocytes less than $10^3$ hapten modified macrophages are required providing that a sufficient number of normal macrophages are present (macrophage help) (Blomberg and Polak, manuscript in preparation). Lymph node lymphocyte suspension contains about 5% macrophages (in our experiments about $2\text{–}4 \times 10^4$). This number would, when modified by the hapten, induce a significant increase in the DNA-synthesis *in vitro*. This was not, however, the case in our experiments. It is suggested that a short pulse with hapten did not induce hapten modified macrophages with corresponding determinants to which antigen reactive lymphocytes induced by an epicutaneous sensitization would respond. This indicates that the *in vitro* responsiveness is dependent mainly on processing and presentation of the hapten by macrophages and to a lesser extent on macrophages help.

The positive results obtained in humans [8, 9] and rabbits [7] with DNCB-Ly suggest, however, that the reacting repertoire may considerably vary between different species.

The notion that contact sensitivity is a T-cell phenomenon was further confirmed by our results. An increased *in vitro* DNA-synthesis of lymphocytes from CY-pretreated animals was confined to this lymphocyte subpopulation. Elimination of B-cells which are suggested to be suppressive in guinea pigs delayed hypersensitivity [16] did not render T-cells responsive to DNCB-Ly preparation. This indicates that the *in vitro* unresponsiveness of lymphocytes from epicutaneously sensitized guinea pigs is not mediated by B-suppressor cells.

To explain the effect of cyclophosphamide pretreatment is more difficult. It is known that application of CY shortly before sensitizing procedure enhances the resulting degree of contact sensitivity [15]. It is assumed that this is due to elimination of suppressor cells. Therefore, the *in vitro* proliferative response of lymphocytes from CY-pretreated animals may be explained by the absence of suppressor cells inhibiting the proliferation of memory cells. However, several arguments contradict the assumption, that the incapability of lymphocytes from epicutaneously sensitized animals of responding *in vitro* to a large set of antigenic preparations is mediated by suppressor cells. At the time when lymphocytes are harvested for DNA-synthesis assay i.e. 17 to 24 days after CY-application, suppressor cells are already regenerated and fully active [15].

On the other hand, elimination of suppressor cells by CY-treatment shortly before an epicutaneous challenge did not enhance the intensity of skin reaction [17]. The same treatment 3 days before cell harvest did not increase the *in vitro* proliferative response to an antigenic stimulation. The stimulation indices in this experiment where 6.6 ± 4.4 compared to 8.0 ± 4.1 in lymphocytes from sensitized animals not pretreated with cyclophosphamide. In this experiment DNP-M$\phi$ were used.

Moreover, addition of lymphocytes from DNCB epicutaneously sensitized guinea pigs did not suppress the enhanced DNA-synthesis of cells from animals sensitized with DNFB in FCA (Table II). Finally, lymphocytes from epicutaneously sensitized animals can be stimulated *in vitro* by DNP-modified macrophages. These results made improbable that suppressor cells are responsible for the failure of lymphocytes from DNCB epicutaneously sensitized guinea pigs to respond in vitro to some antigenic preparations.

It might be that the CY-treatment changed the overall reactivity of the lymphocytes during their regeneration. However, the proliferative response of nonstimulated cells (baseline cpm) was not different in CY-treated and nontreated animals. Furthermore, the *in vitro* stimulation of lymphocytes from CY-pretreated epicutaneously sensitized guinea pigs remained unchanged even when the cells were harvested more than 3 weeks after CY-application.

One may imagine that during the lymphocyte regeneration the diversity of the repertoire of lymphocytes was altered. During this process of generation of diversity, lymphocytes capable of recognizing some self-determinants might be formed. This newly formed repertoire might include also lymphocytes capable of recognizing antigenic determinants formed by the reaction of hapten with these determinants.

Another possibility is that various lymphocyte subpopulations regenerate after CY-treatment to a different degree. Thus, the cell suspensions from CY-pretreated guinea pigs used for the DNA-synthesis assay may contain a higher ratio of antigen reactive cells than suspensions from animals sensitized without CY-pretreatment. Two factors may participate in this effect. (1) Antigenic stimulus in animals, where the lymphoid organs are depleted of lymphocytes might lead to a more potent clonal proliferation and thus to a relatively higher representation of the antigenic specific cells. Since antigen-reactive cells of contact sensitivity are cyclophosphamide-resistant the proliferation of these cells may be favored by the elimination of CY-sensitive lymphocytes. (2) Elimination of suppressor cells at the time of sensitization results in an enhanced formation of specific memory and effectors cells [15].

By these processes the subliminal *in vitro* response of lymphocytes from guinea pigs sensitized epicutaneously with DNCB to minor determinants becomes measurable.

Three factors seem to be decisive for eliciting an enhanced *in vitro* DNA-synthesis: (1) The size of the set of antigens formed during sensitization. (2) The size of the repertoire of lymphocytes capable of recognizing these antigens. (3) The frequency of specific memory cells present in the lymphocyte suspensions.

The practical meaning of this study is that the use of FCA for predictive allergenicity testing in guinea pigs detects larger sensitizing potentials than those active when the substances are applied epicutaneously. The results obtained with the help of adjuvants may therefore to some extent exaggerate the allergenicity risks connected with materials used only epicutaneously. However, under certain pathological circumstances on adjuvant-like effect may occur even during epicutaneous application.

The technical assistance of Mrs. V. Scherer and Mrs. S. Laustsen is gratefully acknowledged.

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